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**INDOLEACETIC ACID OXIDASE AND RELATED ENZYMES IN  
CULTURED AND SEEDLING DOUGLAS-FIR**

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# INDOLEACETIC ACID OXIDASE AND RELATED ENZYMES IN CULTURED AND SEEDLING DOUGLAS-FIR

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## INTRODUCTION

Following the successful asexual propagation of aspen using tissue culture methodology here at The Institute of Paper Chemistry, attention has been turned in recent years to the conifers, particularly Douglas-fir and loblolly pine. The early research resulted in the regeneration of a few aspen trees from callus (an unorganized mass of cells in culture) but not with the desired predictability. When it became apparent that empirical approaches would be insufficient to make this a reliable procedure, a team effort was launched to seek an understanding of biochemical and ultrastructural events associated with morphogenesis leading to plants from cultured tissue.

Callus is generally considered to be totipotent, i.e., to retain all of the genetic potential found in a whole, functioning plant. Nevertheless, not all of this potential is expressed so that cells of a callus mass grow and divide but do not organize into structures such as roots, stems, leaves, etc. The research presented here results from investigations into biochemical differences between callus and the organized tissue from which it originated. If differences can be located at the molecular level, they might then be exploited to reverse the process by which callus was formed. Success in this research could revolutionize tree improvement programs and have great economic impact on the pulp and paper industry.

Indoleacetic acid (IAA) is an important plant hormone; it is also known as natural auxin. For many years there has been a major hypothesis stating essentially that the ratio of auxins to other types of plant hormones in a system determines the course of plant development. In this report we focus on the enzymatic destruction of IAA in callus versus organized parts of seedlings. The

results suggest that some callus may become locked in that unorganized state because of deficiencies in the capacity to degrade endogenous IAA.

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Running Title: IAA Oxidase in Douglas-fir

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Key Term Index: IAA oxidase, peroxidase, catalase, superoxide dismutase,  
scopoletin, Pseudotsuga menziesii

## SUMMARY

Anionic electrophoretic banding patterns for isozymes of IAA oxidase and peroxidase from Douglas-fir needle, stem, and hypocotyl callus were compared with patterns from seedling needles, stems, and hypocotyls-roots. The seedling sections yielded characteristic patterns which ranged from complex (hypocotyls-roots) to relatively simple (stems). Callus patterns tended to reflect the tissue of origin but fluctuated during culture. All IAA oxidase isozymes exhibited peroxide dependence as resolved on polyacrylamide gels; however, spectrophotometric examination of total (unresolved) IAA oxidase activity revealed only a lag phase in the absence of added hydrogen peroxide. The IAA oxidase isozymes corresponded to isoperoxidases in terms of electrophoretic mobility. Their on-gel activity can be modified by the presence of phenolics such as scopoletin. Catalase and superoxide dismutase also were extracted from callus where they may participate in the control of peroxide levels. It is postulated that regulation of peroxide concentration, possibly in concert with the influence of endogenous phenolics, is a major determinant of IAA oxidase activity in these tissues.

## INTRODUCTION

Many investigators have studied isoperoxidases as indicators of plant growth and development (e.g., VERMA and VAN HUUSTEE 1969, MÄDER et al. 1975). One approach to assessing the role of isoperoxidases in plant development has been to compare isozyme patterns of extracts of organized tissues obtained from normal plants with those of extracts from unorganized callus (SHINSHI and NOGUCHI 1976, BAJAJ et al. 1973). Such strategy was adopted in our laboratory for several enzymes to pinpoint certain peroxide-related molecular events during organogenesis or embryogenesis from conifer callus or suspension cells (WINTON et al. 1974).

BLIGNY et al. (1976) found that, under shoot-producing conditions, extracts of cultured Jerusalem artichoke exhibited three high mobility anionic isoperoxidases which were not present under root-producing conditions as controlled by naphthaleneacetic acid concentrations. SIMON et al. (1974) concluded that peroxidase, catalase, and superoxide dismutase activities fluctuate in a periodic manner on a fresh weight basis during bean plant development. Root formation from carrot callus has been linked to the production of new forms of polyphenol oxidase (HABAGUCHI 1977a, 1977b). Polyphenols are produced in response to cut injury (TANAKA and URITANI 1977). Since cutting is a common operation in preparing explants for callus initiation, the observations of BORCHERT (1974) and of ZIMMERMANN and ROSENSTOCK (1976) regarding wound-induced isoperoxidases might also be relevant to enzyme activities in the callus state. It seems to us that wound induction of enzymes at the time of callus initiation may be perpetuated in culture and constitute a serious barrier to subsequent efforts to regenerate plants.

The catabolism of indole-3-acetic acid (IAA) has been studied in some cultured tree species. Auxin independence in a cultured sycamore strain was

not directly traceable to IAA oxidase in the investigations of MAILLARD et al. (1976). However, dark-grown "Shamouti" orange embryogenic callus was shown to decompose IAA at a faster rate than nonembryogenic callus (EPSTEIN et al. 1977). The "Shamouti" orange embryogenic callus is characterized by high total peroxidase activity and a unique cationic isoperoxidase (KOCHBA et al. 1977).

It was observed that, relative to the originating tissue, most tree callus (several species in our laboratories) was characterized by the presence of substantial catalase activity (unpublished). Because catalase and peroxidase may compete for available hydrogen peroxide and catalases usually have peroxidatic activity (SAUNDERS et al. 1964a, HALLIWELL 1974), these enzymes were investigated together. Initial correlations of isoperoxidase electrophoretic patterns with enzyme source and the well-known strong relationship between IAA oxidase and peroxidase (GOVE and HOYLE 1975) made investigation of IAA oxidase imperative. Since our attempts to promote embryoid formation based upon countering catalase and promoting IAA oxidase in unorganized suspension cultures have been somewhat promising (JOHNSON and CARLSON 1977), it was hypothesized that control of the endogenous peroxide concentration could be crucial to development in this system. OMRAN (1977) recently proposed direct reversible chemical inactivation of IAA by hydrogen peroxide via complex formation. The foregoing enzymes and any others involved in peroxide production, utilization or destruction in vivo may also have important regulatory roles of which auxin degradation may be only one possibility. Superoxide dismutase is a prime example of a related enzyme, and some data on its presence in this system are presented also. Our emphasis on natural auxin in this report is not meant to imply that other plant hormones have no role in conifer development.

## MATERIAL AND METHODS

### MATERIAL

The Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] callus was grown in our associated tissue culture laboratory; details of the tissue culture methodology have been described elsewhere (WINTON 1972, WINTON AND VERHAGEN 1977). Where relevant, further details on the cultured tissues used are provided with the results, but it should be noted that normally subculturing on agar is on a monthly basis. Analyses of organized tissues were performed on parts of Douglas-fir seedlings up to six months of age that were grown from seed in soil in a greenhouse.

### ENZYME EXTRACTION

All enzyme preparations were extracts of acetone powders of callus or seedling sections. Powders of the various tissues were immediately extracted with 100 mM morpholinopropane sulfonic acid (MOPS) buffer, pH 7.0, insoluble residue being removed by centrifugation. These extracts were then passed through gel filtration columns of BioGel P-2 equilibrated with the same buffer but at 10 mM, and the excluded peak was taken for electrophoresis or other analyses of enzymes. Protein was determined by a microbiuret procedure (KOCH and PUTNAM 1971).

### ELECTROPHORESIS AND ON-GEL STAINING OF ENZYMES

A standard anionic disc electrophoresis procedure (BREWER and ASHWORTH 1969) was employed for the isozyme analysis; samples were loaded in sucrose solution and no spacer gel was used. Extract loads of 0.2 ml (10-30 µg protein from organized tissues; 100-200 µg protein from callus tissues) were electrophoresed for 2 h at 2 mA per tube on preelectrophoresed gels. Peroxidase (EC 1.11.1.7) activity on gels was located by the procedure of



GREGORY and FRIDOVICH (1974) for catalase using diaminobenzidine as substrate but eliminating the horseradish peroxidase. IAA oxidase was located by the procedure of ENDO as described by SRIVASTAVA and VAN HUUSTEE (1973). No peroxidase bands were observed in the absence of diaminobenzidine nor IAA oxidase bands in the absence of IAA. The catalase (EC 1.11.1.6) banding pattern presented was obtained by a negative staining procedure (VERMA and VAN HUUSTEE 1970). On-gel staining for superoxide dismutase (EC 1.15.1.1) was according to BOHNENKAMP and WESER (1975). Stained gels were scanned with an ISCO gel scanner. Although no data is presented here, extra samples were routinely prepared and stained for the total protein pattern with Coomassie Brilliant Blue.

#### SPECTROPHOTOMETRIC DETERMINATION OF UNRESOLVED IAA OXIDASE

Conditions for this determination were adapted from the on-gel staining procedure except that up to 0.5 ml of BioGel P-2 excluded extracts was incubated at 25°C in a total volume of 3.0 ml and the Fast Blue BB dye was eliminated. After an initial scan from 240 to 300 nm without extract, the reaction was started by enzyme addition and repetitively scanned at 4 min intervals in a Perkin-Elmer Model 576 spectrophotometer against a double-distilled water reference. Controls lacking substrate, enzyme, and other assay constituents were prepared and scanned in the same manner. Similar procedures have been used by others (e.g., RITZERT et al. 1972, LEE 1972) to follow the formation of the methyleneoxindole oxidation product at 253 nm.

### RESULTS AND DISCUSSION

#### VARIABILITY OF ISOZYME PATTERNS

Culture parameters considered as possible contributors to variability of isozyme patterns obtained from callus extracts were: growth medium,

clone, the number of subcultures (i.e., related to the age of the tissue in the cultured state) and the time of assay relative to the most recent subculture (hereafter referred to as subculture age). Of these four factors, the subculture age was the only one found to have significant impact at the level of resolution considered here. The effect of subculture age is illustrated by the anionic isoperoxidase banding data in Fig. 1 for extracts of Douglas-fir stem callus that had been in culture a long time. A periodicity was observed in which a fast-moving peak region dominated the pattern for a time following subculture but then declined. Normal subculturing reactivated this peak region and the process repeated. This rejuvenation has been observable within one day of subculture (Fig. 2A). In an extract of callus kept untouched for 79 days on the same agar before assay, this peak region was diminished relative to its size in an extract of callus of essentially the same age which had been subcultured twice during this time (Fig. 2B). This is not simply a matter of the unsubcultured tissue running out of nutrients and deteriorating, for the same difference was noted (Fig. 2C) when callus was transferred to fresh medium without subdivision at the same time that the identical callus was subcultured normally, maintaining approximately the same callus/agar ratio in each case. Since this callus is of a rather soft texture, subdivision at the time of subculture results in little mechanical damage; therefore, the major effect of subdivision may be aeration of more callus surface. PENEL et al. (1977) noted that isoperoxidase banding patterns are subject to modification by ionic strength, pH, temperature, and associated compounds during extraction. A standardized procedure was used for all extractions in our investigations. An additional factor requiring standardization is the time of color development in the on-gel enzyme staining procedure as there can be differential rates of color development in band regions.

[Fig. 1-2 here]

We present the banding pattern comparisons that follow fully cognizant of possible perturbations of isozyme patterns such as discussed above. These banding distributions are reproducible in terms of major peak regions but not necessarily in fine detail.

#### PEROXIDASE AND IAA OXIDASE

Anionic distribution patterns of isoperoxidases from extracts of needle, stem, and hypocotyl callus are compared with IAA oxidase patterns from the same extracts in Fig. 3. Isozymes of peroxidase and IAA oxidase from these cultured tissues can be grouped into three regions of anionic electrophoretic mobility. Each region exhibits heterogeneity. Where IAA oxidase isozymes appear, they correlate well with isoperoxidases in terms of electrophoretic mobility. Although the entire pattern from stem and hypocotyl callus has slightly increased mobility, it seems to consist of the same three major peak regions (supported also by consideration of Fig. 4 and 5 below).

[Fig. 3-5 here]

Activities of peroxidase and IAA oxidase isozymes were also examined in extracts of organized tissues, i.e., from different parts of greenhouse seedlings. A distinct anionic isoperoxidase pattern was associated with each of the seedling sections: needles, stems, and hypocotyls-roots. Patterns are shown in Fig. 4 as elicited in the presence and absence of added hydrogen peroxide. Peaks appearing in the absence of added peroxide may be indicative of polyphenol oxidase (EC 1.10.3.1) activity with similar mobility or even residing in the same protein species as suggested by the results of SRIVASTAVA and VAN HUUSTEE (1973, 1977). The IAA oxidase patterns obtained from the same preparations at the same time (Fig. 5) again show considerable correspondence with the isoperoxidases. Despite their different origins and a tendency to reflect isozyme patterns characteristic of the originating plant organ (Fig. 4

and 5), all of the callus patterns (Fig. 3) appear to contain essentially the same complement of isozymes; however, the activity of any individual isozyme is expressed in varying degrees. In the organized originating tissue (Fig. 4 and 5) these expressions become fixed in the different sections of the seedling during development whereas in culture the expression of activity of an individual isozyme may change (e.g., previous discussion on the effect of subculture age).

Although there is strong association with peroxide in data presented here, the oxidizing agent in IAA oxidase activity is not generally considered to be peroxide, but it is often added as a promoter and is essential for maximum initial velocities (HOYLE and ROUTLEY 1974). It may be notable, therefore, that IAA oxidase isozymes from various parts of the seedlings as well as from callus (Fig. 6) show complete dependence upon peroxide addition. Nevertheless, spectrophotometric examination of total IAA oxidase activity in unfractionated extracts of needle callus revealed only a lag phase in the absence of added peroxide (Fig. 7d). As can be seen from the other spectra in Fig. 7, these extracts apparently were capable of oxidizing the trichlorophenol cofactor to a product absorbing at 273 nm when IAA was omitted. It was also observed that manganese ion was not necessary in the assay when peroxide was added. However, in the absence of both exogenous peroxide and manganese ion there was no activity. During the lag phase, observed when only peroxide is omitted, peroxide may be synthesized enzymatically via oxidase activity on the trichlorophenol or even chemically via manganese-catalyzed oxidation of some bound endogenous phenolic (SZENT-GYÖRGYI 1972, SAUNDERS et al. 1964b).

[Fig. 6-7 here]

Scopoletin, which has been reported to be both an IAA oxidase inhibitor and promoter depending upon concentrations used (IMBERT and WILSON 1970),

enhanced development of IAA oxidase bands on the gels. While the control shows that scopoletin is not giving rise to the peaks, there is general IAA oxidase isozyme activation in the data of Fig. 8. Notable is the effect on the high mobility band region which had essentially disappeared in this needle callus. Resurgence of activity with scopoletin treatment shows that these isozymes were still present but inactive. These results are similar to the report of SCHAFER et al. (1971) in which scopoletin specifically stimulated a high mobility anionic isoperoxidase from tobacco callus.

[Fig. 8 here]

#### CATALASE AND SUPEROXIDE DISMUTASE

Callus catalase, when present, is easily demonstrated visually in vivo by injecting the callus with weak peroxide solution or quantitatively in vitro according to BEERS and SIZER (1952) (unpublished). This activity also appears to be associated with low mobility isoperoxidases during anionic electrophoresis (Fig. 6). Whether it accounts for some of the observed isoperoxidatic activity in this system is not clear but it appears likely. Catalase is difficult to demonstrate in organized tissues of Douglas-fir except for weak activity at cut surfaces, which, incidentally, is where callus is originated. Recent reports indicate that horseradish peroxidase is able to generate hydrogen peroxide (ELSTNER and HEUPEL 1976) and superoxide radicals (HALLIWELL and AHLUWALIA 1976). The latter ( $O_2^{\bullet -}$ ) is rendered harmless by conversion to  $O_2$  and  $H_2O_2$  by superoxide dismutase thereby avoiding the production of singlet oxygen in biological systems. Activity of superoxide dismutase can also be demonstrated following electrophoresis of stem callus extracts (Fig. 9). Again the pattern is seen to vary with subculture age. The significance of catalase and superoxide dismutase in callus remains under investigation.

However, these callus tissues are well-equipped for catabolism involving peroxide. One function for the peroxide in developmental processes may relate to its role in IAA oxidase activity, particularly relative to isozymes with high anionic mobility in this system.

[Fig. 9 here]

#### POTENTIAL OF IAA OXIDASE AS A DEVELOPMENTAL MARKER

Our investigations show that while isoperoxidases of Douglas-fir exhibit distribution patterns characteristic of the originating plant organs, IAA oxidase isozymes do also and may present additional opportunities for interpretation due to the importance of the natural substrate. Binding of low molecular weight compounds to these enzymes in vitro or in vivo may be very significant as exemplified by the effect of scopoletin (Fig. 8). As indicated by the scopoletin response and Fig. 3-5, it appears that all isozymes may be in all parts of the plant but differentially active in different organs. Regulatory effects of phenolic compounds on IAA oxidase have been discussed by several authors over a number of years (e.g., PINGEL 1976). PENEL *et al.* (1977) pointed out that binding of phenolics and other small molecules to peroxidases during extractions might account for some perturbation of isozyme patterns which they observed.

The appearance of the peroxide dependence of the Douglas-fir isozymes following resolution further suggests that knowledge of localization and of the changing microenvironment of these isozymes during development might be of value in determining their roles in morphogenetic events. We are attempting to relate changes in these isozyme patterns to the morphogenetic potential of cultured tissue. Our attention has been drawn to high mobility anionic isozymes which are useful in distinguishing seedling section patterns, are peroxide

dependent, and are susceptible to change during culture. The persistence of activity in the high mobility band region has been observed to correlate with shoot production from callus in some cases; the IAA oxidase and peroxidase isozyme patterns in Fig. 6 represent one example.

#### ACKNOWLEDGMENT

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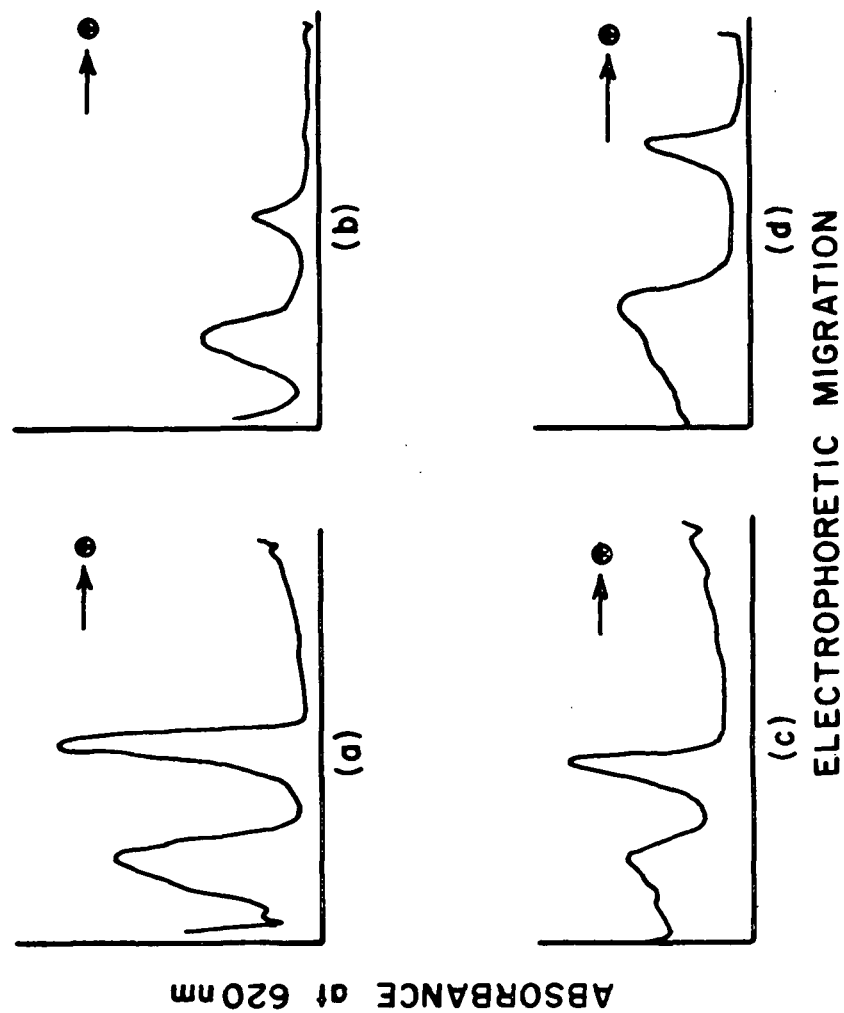


Fig. 1. Electrophoretic distribution of stem callus peroxidase as a function of the number of subcultures and subculture age: (a) 17 days after the 61st subculture, (b) 24 days after the 61st subculture, (c) 14 days after the 63rd subculture, (d) 27 days after the 63rd subculture.

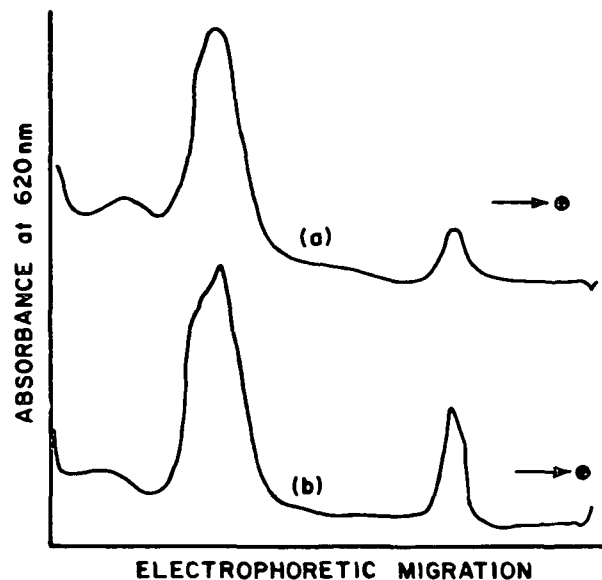
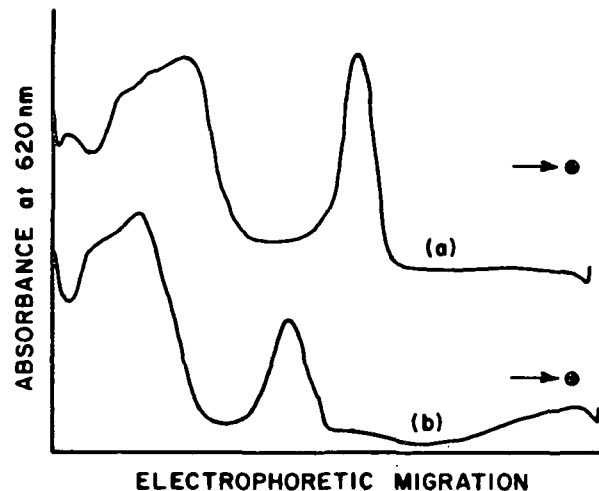
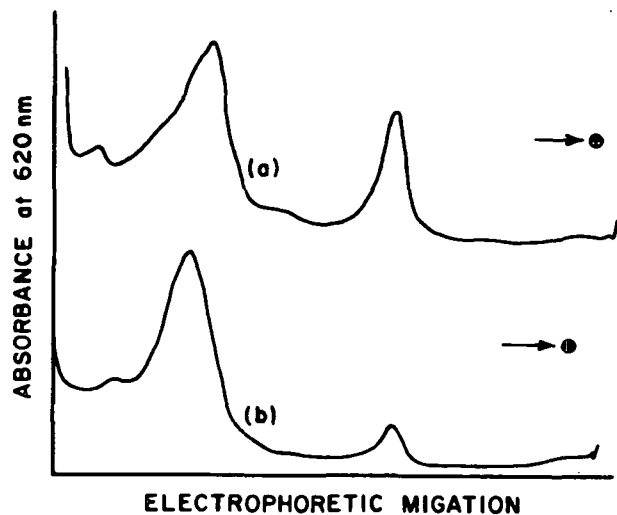


Fig. 2A. Rapid change in the isoperoxidase banding pattern: (a) one day after subculture onto the same medium, (b) identical with (a) but unsubbed.

Fig. 2B. Effect of prolonged growth of callus on agar without subculture on the isoperoxidase banding pattern: (a) 21 days after the 59th subculture, (b) 79 days after the 57th subculture.

Fig. 2C. Effect of subdivision of callus at the time of subculture on the isoperoxidase banding pattern: (a) whole callus pieces transferred during two subcultures, (b) callus normally subcultured with subdivision otherwise identical to (a).

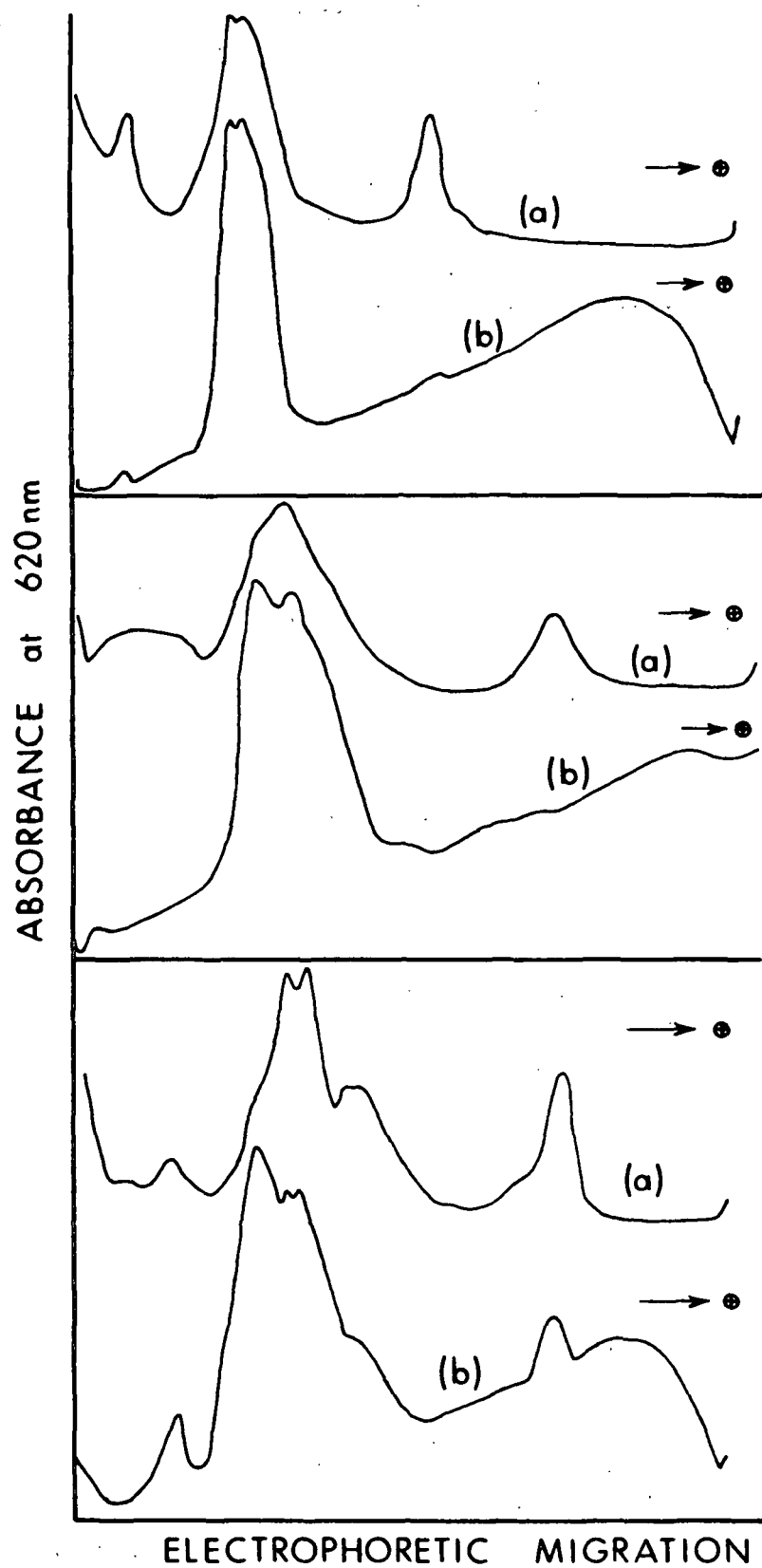


Fig. 3. Isozyme banding patterns for peroxidase (a) and IAA oxidase (b)  
extracted from: A. Needle Callus - subculture age - 19 days  
 B. Stem Callus - subculture age - 22 days  
 C. Hypocotyl Callus - subculture age - 4 days.

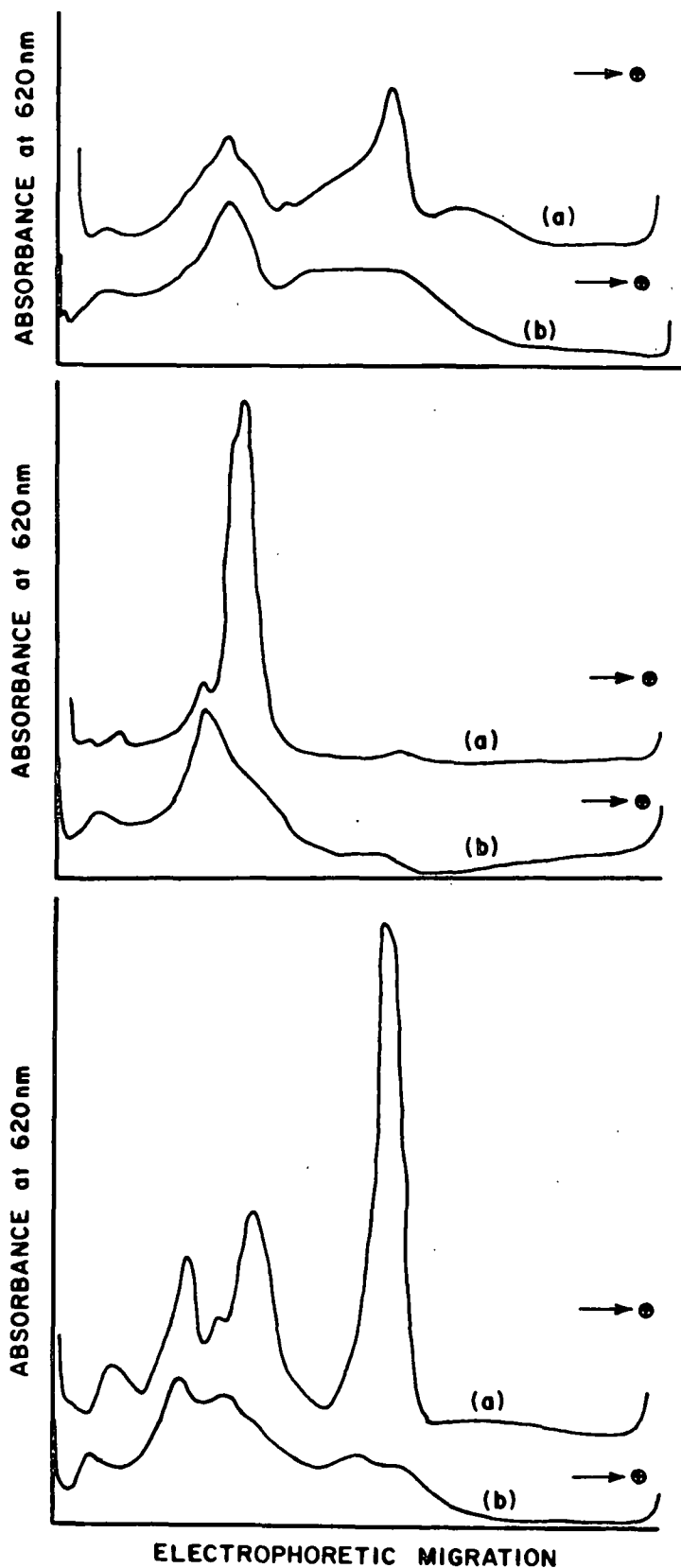


Fig. 4A. Isoperoxidase banding pattern of needles with (a) and without (b) added  $H_2O_2$ .

Fig. 4B. Isoperoxidase banding pattern of stems with (a) and without (b) added  $H_2O_2$ .

Fig. 4C. Isoperoxidase banding pattern of hypocotyls - roots with (a) and without (b) added  $H_2O_2$ .

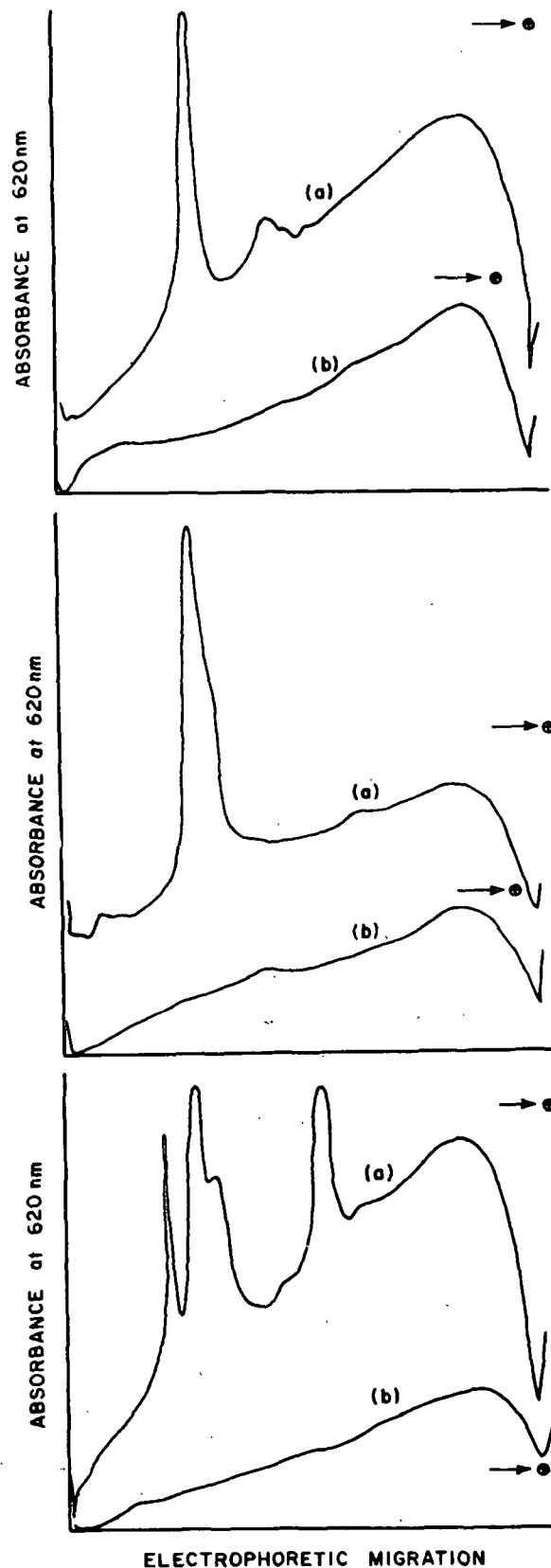


Fig. 5A. IAA oxidase isozyme banding pattern of needles with (a) and without (b) added  $H_2O_2$ .

Fig. 5B. IAA oxidase isozyme banding pattern of stems with (a) and without (b) added  $H_2O_2$ .

Fig. 5C. IAA oxidase isozyme banding pattern of hypocotyls - roots with (a) and without (b) added  $H_2O_2$ .

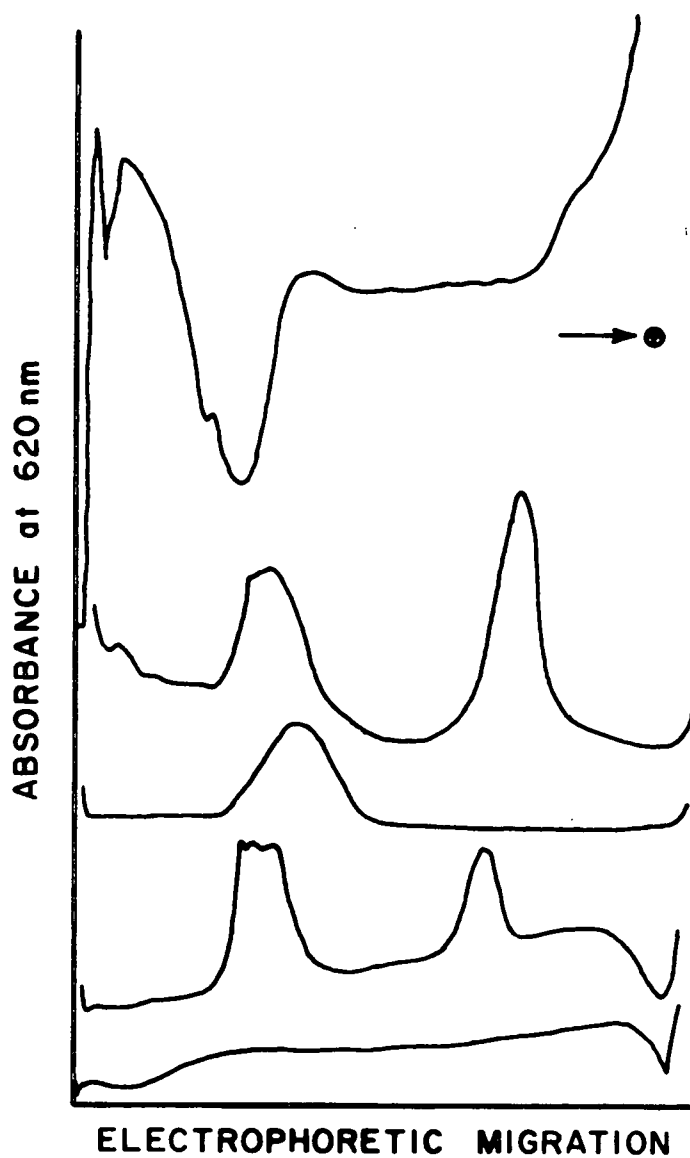


Fig. 6. Isozyme banding patterns of enzymes extracted from needle callus (subculture age: 23 days). Top to bottom: Catalase (negative peak), peroxidase, peroxidase without added  $H_2O_2$ , IAA oxidase, IAA oxidase without added  $H_2O_2$ .



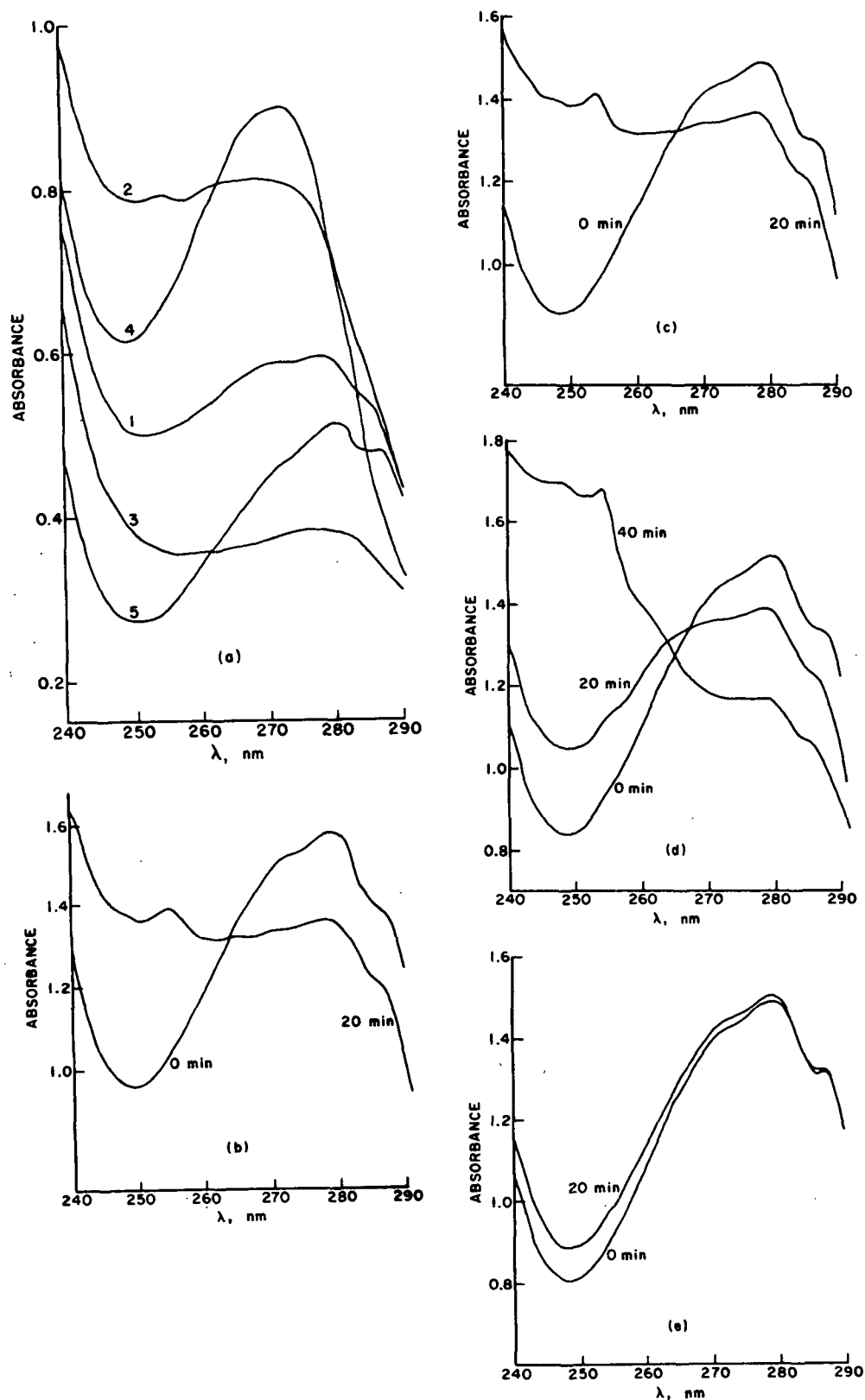


Fig. 7. Progression of IAA oxidation catalyzed by unresolved needle callus extract: (a) extract and IAA dependence: curve 1 - zero time complete system, curve 2 - 8 min complete system, curve 3 - zero time without IAA, curve 4 - 8 min without IAA, curve 5 - zero time and 8 min without extract (buffer only); (b) complete system: (c) without  $Mn^{+2}$ ; (d) without added  $H_2O_2$ ; (e) without  $Mn^{+2}$  and added  $H_2O_2$ .

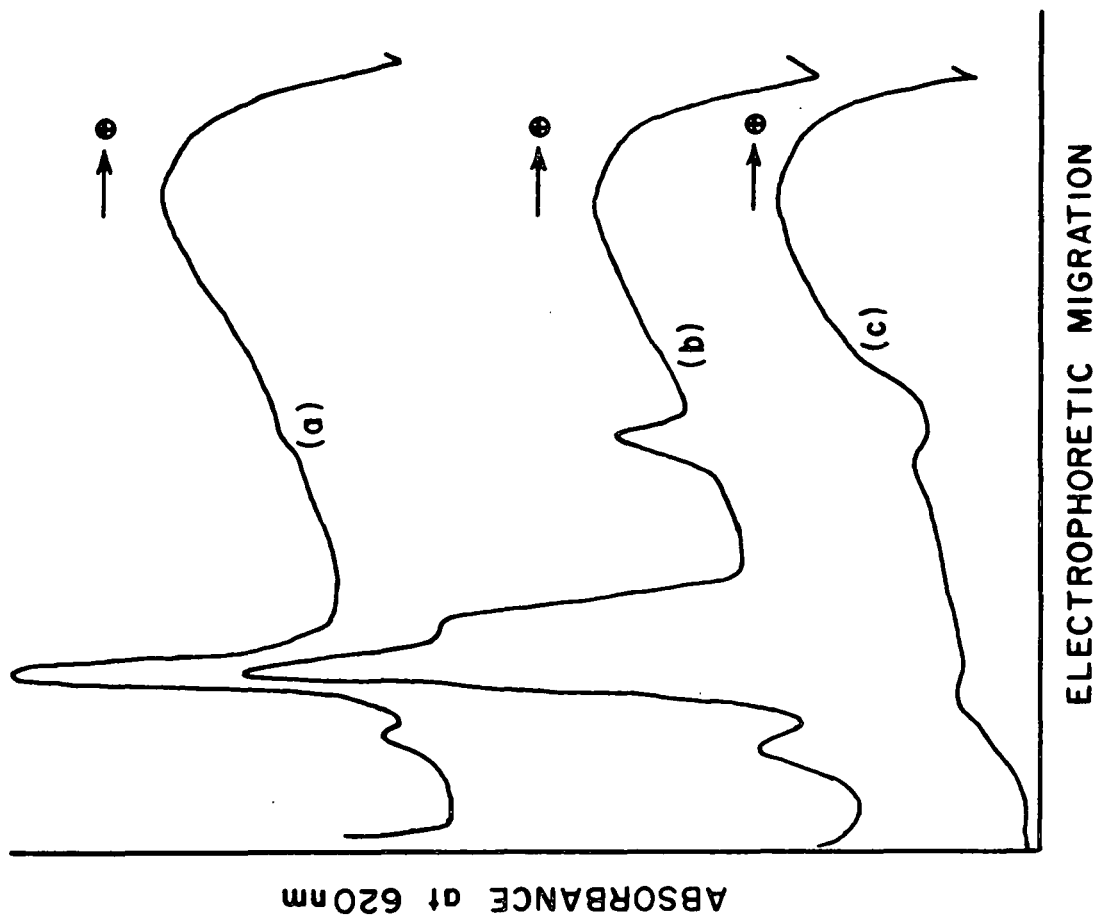


Fig. 8. Effect of the presence of scopoletin during on-gel staining for IAA oxidase: (a) control - complete system, (b) in the presence of 50  $\mu$ M scopoletin, (c) as in (b) without IAA present.

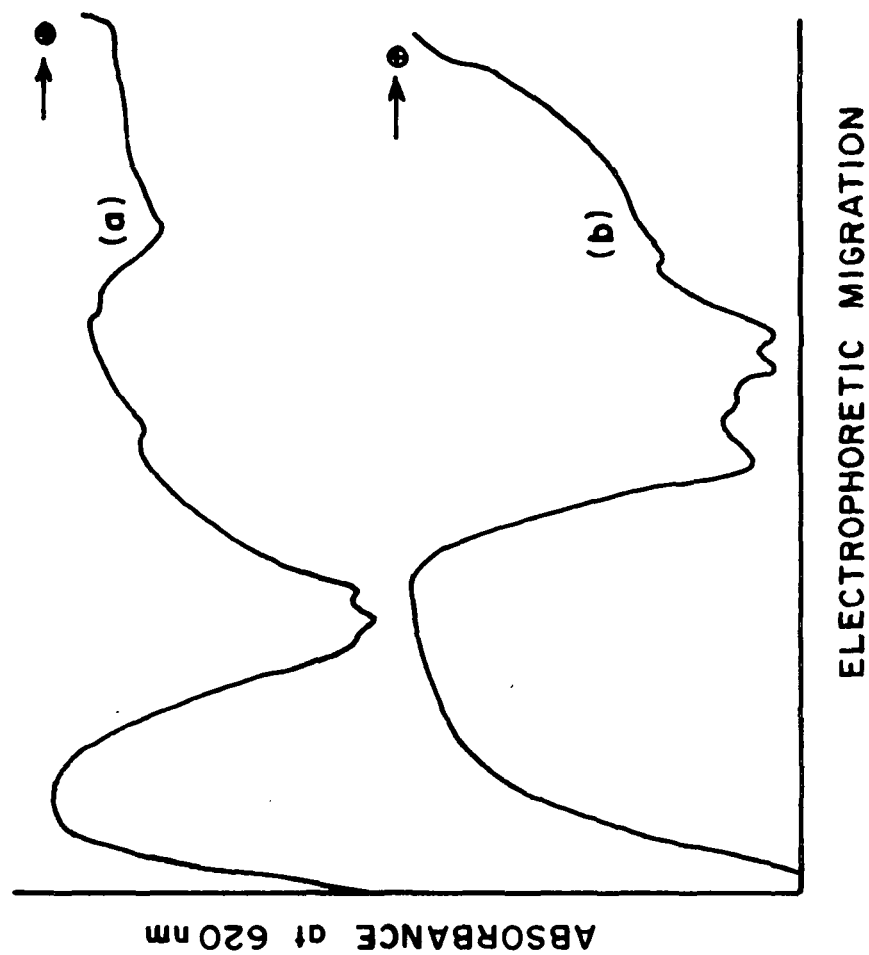


Fig. 9. Superoxide dismutase (negative peaks) from stem callus: (a) 14 days after subculture, (b) 25 days after subculture.